Laminin Chains: Diagnostic and Therapeutic Use

Background of the Invention

Laminins are a family of basement membrane proteins which function in cell differentiation, adhesion, and migration, in addition to being true structural components (Tryggvason K, *Curr. Opn. Cell Biol.*, 1993, **5**:877-882, this and all following references are hereby incorporated by reference). The laminin molecule is a cross-shaped heterotrimer consisting of one heavy α chain (~400 kd) and two light chains, ß and γ (130-200 kd) (nomenclature according to Burgeson et al., *Matrix Biol.*, 1994, **14**:209-211). Laminin exists in numerous isoforms that are formed by different combinations of laminin chain varients which currently amount to at least nine.

Kalinin/laminin 5 (most likely also identical to the adhesion molecule nicein) is a recently identified laminin isoform which is a functional adhesion component for epithelial cells (Tryggvason, 1993, *supra.*; Burgeson et al., 1994, *supra.*; Rousselle et al., *J. Cell Bio.*, 1991, **114**:567-576; Kallunki et al., *J. Cell Biol.*, 1992, **119**:679-693; Marinkovich et al., *J. Biol. Chem.*, 1992, **267**:17900-17906; Vailly et al., *Eur. J. Biochem.*, 1994, **219**:209-218). Kalinin/laminin 5 contains unique laminin varient chains, one of which, the γ2 chain, has recently been cloned and sequenced (Kallunki et al., 1992, *supra.*, previously named B2t). The γ2 chain has a mass of ~130 kd and is thus smaller than the "classical" ~200 kd ß1 and γ1 light chains of laminin. The domain structure of the γ2 chain also differs from that of the γ1 chain in that it lacks the amino-terminal globular domain (domain VI) believed to function in intermolecular cross-linking of laminin molecules to form networks (Yurcheno and O'Rear, in Molecular and Cellular Aspects of Basement Membranes, 1993, (ed. Rohrbach and Timpl, Academic Press, San Diego, pp. 20-47). In addition, domains III, IV, and V (containing EGF-like repeats) in γ2 are shorter than in the γ1 chain (Kallunki et al., 1992, *supra.*).

By *in situ* hybridization the γ2 chain was found to be expressed in epithelial cells of many embryonic tissues such as those of skin, lung, and kidney (Kallunki et al., 1992, *supra.*), and antibodies to kalinin/laminin 5, react with basement membranes of the same tissues (Rousselle et al., 1991, *supra.*; Verrando et al., *Lab. Invest.*, 1991, **64**:85-92).

The different laminin chains have been shown to have quite varying tissue distribution as determined by immunohistological studies, Northern, and *in situ* hybridization analyses. For example, the A and M chains on the one hand, and the B1 (ß1) and S (ß2) chains on the other, have been shown to be mutually exclusive (see for example Vuolteenaho et al., *J. Cell Biol.*, 1994, **124**:381-394). In vitro studies have indicated that laminin mediates a variety of biological functions such as stimulation of cell proliferation, cell adhesion, differentiation, and neurite outgrowth. The cellular activities are thought to be mediated by cell memebrane

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receptors, many of which are members of the integrin family (Ruoslahti, E. *J. Clin. Invest.*, 1991, **87**:1-5; Mecham, R.P. *FASEB J.*, 1991, **5**:2538-2546; Hynes, R. *Cell*, 1992, **69**:11-25).

Recently a new nomenclature for describing laminins has been agreed to as in the following Table 1 (after Burgeson et al., 1994, *supra.*)

Table 1

laminin chains and genes			heterotrimers of laminin		
New	Previous	Gene	New	Chains	Previous
α1	A, Ae	LAMA1	laminin-1	α1β1γ1	EHS laminin
α2	M, Am	LAMA2	laminin-2	α2β1γ1	merosin
α3	200 kDa	LAMA3	laminin-3	α1β2γ1	s-laminin
β1	B1, B1e	LAMB1	laminin-4	α2β2γ1	s-merosin
β2	S, B1s	LAMB2	laminin-5	α3β3γ2	kalinin/nicein
β3	140 kDa	LAMB3	laminin-6	α3β1γ1	k-laminin
γ1	B2, B2e	LAMC1	laminin-7	α3β2γ1	ks-laminin
γ2	B2t	LAMC2			

Summary of the Invention

The instant invention provides for methods of detecting kalinin/laminin 5 expression in tissue comprising detecting a signal from assayed tissue, such signal resulting from specifically hybridizing tissue with an effective amount of a nucleic acid probe, which probe contains a sense or antisense portion of kalinin/laminin 5 gamma-2 nucleic acid sequence (Kallunki et al., 1992, *supra.*). In particular, where the nucleic acid probe is DNA, RNA, radiolabelled, enzyme labelled, chemiluminescent labelled, avidin or biotin labelled, derived from human kalinin/laminin 5 gamma-2 nucleic acid sequence, incorporated into an extrachromasomal self-replicating vector, a viral vector, is linear, circularized, or contiains modified nucleotides. In the preferred embodiment the probes are linearized specific regions of the γ2 gene.

The instant invention also provides for methods for detecting the presence of invasive cells in tissue comprising detecting a signal from assayed tissue, such signal resulting from contacting tissue with an effective amount of a nucleic acid probe, which probe contains a sense or antisense portion of kalinin/laminin 5 gamma-2 nucleic acid sequence (Kallunki et al., 1992, *supra.*). In particular, where the nucleic acid probe is DNA, RNA, radiolabelled, enzyme labelled, chemiluminescent labelled, avidin or biotin labelled, derived from human kalinin/laminin 5 gamma-2 nucleic acid sequence, incorporated into an extrachromasomal self-replicating vector, a viral vector, is linear, circularized, or contiains modified nucleotides. In the preferred embodiment the probes are linearized specific regions of the γ2 gene. The instant method also provides for the diagnosis of the absence of γ2 chain expression, useful for the

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monitoring of therapies, and the progress of malignant cell transformation leading to accurate determination of the extent of invasive cell activity.

The instant invention futher provides for a method for detecting kalinin/laminin 5 expression in tissue comprising detecting a signal from assayed tissue, such signal resulting from contacting tissue with an effective amount of a labeled probe, which probe contains an antibody immunoreactive with a portion of kalinin/laminin 5 gamma-2 protein.

Further provided is a method for detecting invasive cells in tissue comprising detecting a signal from assayed tissue, such signal resulting from contacting tissue with an effective amount of a labeled probe, which probe contains an antibody immunoreactive with a portion of kalinin/laminin 5 gamma-2 protein. Also provided is a method for detecting kalinin/laminin 5 in tissue comprising detecting a signal from assayed tissue, such signal resulting from contacting tissue with an effective amount of a labeled probe, which probe contains an antibody immunoreactive with a portion of kalinin/laminin 5 gamma-2 protein. Thus the method of the instant invention provides for the absence of such signal as diagnostic for the absence of invasive cells.

Brief Description of the Drawings

Figure 1 shows In situ hybridization of a specimen of colon adenocarcinoma for γ2 chain mRNA using a S-35 labeled anti-sense RNA probe derived from plasmid pbb2r-02. Magnification: 1A x 100; 1B-1D x 640.

Figure 2 shows In situ hybridization for γ2 chain mRNA on sections of ductal mammary carcinoma (2A), malignant melanoma (2B), squamous cell carcinoma of the skin (2C-2D), and squamous cell carcinoma of the vulva (2E-2G). Magnification: 2C x 100, all others x 640.

Figure 3 is incisionally wounded mouse skin (72 hours after wounding) showing signal for γ2 chain in keratinocytes at the leading edge of the migrating epithelium (curved arrow). Magnification: x 640.

Figure 4 shows the nucelic acid sequence for the γ2 chain cDNA and the derived amino acid sequence. Figure 4A is the full cDNA for the 5,200 base pair sequence, availible from EMB/GenBank/DDBJ under the accession number Z15008. Figure 4B is the nucleotide and derived amino acid sequence of the alternative 3' end sequence from cDNA clones providing a sequence of 4,316 base pairs, availible from EMB/GenBank/DDBJ under the accession number Z15009. (Kallunki et al., 1992, *supra.*).

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Detailed Description of the Invention

Epidermolysis bullosa (EB) is a group of mechano-bullous disorders characterized by fragility of the skin and mucous membranes (see Lin & Carter eds., Epidermolysis bullosa, Basic and clinical aspects, 1992, Springer Verlag, N.Y.; Fine et al., *J. Am. Acad. Dermatol.*, 1991, 24:119-135). The junctional forms of EB (JEB) are characterized by tissue separation at the level of the lamina lucida within the dermal-epidermal basement membrane, and no specific mutation had yet to be reported. Recently it has been proposed that the genes for a lamina lucida protien kalinin/nicein/epiligin may be a candidate in some forms of JEB (Verrando et al., 1991, *supra.*). Several lines of evidence suggest that anchoring filament proteins could be defective in some forms of JEB. First, attenuation or absence of immunoreactivity with anti-kalinin(epiligrin) antibodies has been noted in the skin of patients with the most severe (Herlitz) type of JEB. The immunofluorescence staining patterns may be of prognostic value in classifying JEB, and these immunoreagents have been used for prenatal diagnosis of JEB using fetal skin biopsy specimins. Second, the kalinin/laminin 5 $\gamma 2$ chain is expressed in epithelial cells of the skin, trachea and kidneys, tissues which are frequently affected by JEB.

Since the majority of cases are of the generalized (Herlitz) phenotype (H-JEB), JEB patients have been classified into Herlitz and non-Herlitz types. Clinical features of H-JEB include mechanical fragility of the skin, with widespread blistering and erosions, rapid deterioration and neonatal death, often from sepsis. Longtern survival is rare.

Efforts to identify the basic defect in JEB began with the observation that a monoclonal antibody that binds to the lamina lucida of the epidermal basement membrane zone of normal skin, fails to react with the lamina lucida of H-JEB skin (Verrando et al., 1991, *supra.*). The antigen recognized by this antibody was purified from keratinocyte culture medium and termed BM600/nicein. Keratinocytes cultured from the skin of H-JEB patients attach poorly to substrate and fail to accumulate immunologically detectable nicein. Further experiments with antibodies specific for the $\alpha 3$ chain of nicein, demonstrated that they were capable of inducing the rounding and detachment of adherent keratinocytes without affecting fibroblasts (Rousselle et al., 1991, *supra.*). Thus the correlation *in vivo* and *in vitro* of the dermoepidermal separation with deficient nicein/kalinin/laminin 5 immunoreactivity and the separation induced by anti-nicein antibody have made the genes encoding this protein strong candidates for the site of H-JEB mutations.

The importance of the $\gamma 2$ chain of nicein/kalinin/laminin 5 in JEB, and epithelial tissues prompted the investigation into the role such adhesion contacts between epithelial cells may

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play in abberant cells. Of primary interest was the role γ2 chain of nicein/kalinin/laminin 5 abberant expression may play in cancer tissue, and a possible role in cancer dissemination.

It has been recently shown that in colon adenocarcinoma, a significant positive correlation between the degree of tumor budding and the recurrence of tumors following curative surgery exists, and that this fact is likely to reflect a higher invasive potential of budding cancer cells as compared with cancer cells located deeper in the tumor (Hase et al., *Dis. Colon Rectum*, 1993, 36:627-635). Therefore, as demonstrated in Example 3 below, the instant invention allows for the useful prognostic determination of success of surgery, means for monitoring progression of tumor budding and subsequent prognosis.

The identification of the role of γ2 chain allows for the novel use of kalinin/laminin 5 γ2 chain and its ligand, as diagnostic probes of the tumor cell/basement membrane adhesion interface that is crucial for the invasion of non-malignant tissues, and identifies invasive cells.

Thus the identification of the role of γ2 chain allows for the novel therapeutic intervention of binding of kalinin/laminin 5 to its ligand, and thereby reducing the tumor cell/basement membrane adhesion that is crucial for the invasion of non-malignant tissues, and method for inhibiting the budding of tumor masses, and a means for determing the level of γ2 chain expression as a measure of budding activity of a given tumor.

As demonstrated in Example 3 below, the γ2 chain of kalinin/laminin 5 is preferentially expressed by invasively growing malignant cells in human carcinomas. Furthermore, migrating keratinocytes in wound healing also expressed this gene, pointing to a role of γ2 chain in epithelial cell migration both in malignant and in nonmalignant pathological conditions. The consistent expression of the γ2 chain gene in invading cancer cells reflects a functional importance of this molecule *in vivo* in establishing contacts between the invading malignant cells and a provisional matrix in the immediate surroundings of the cancer cells. The instant invention provides methods for the identification of, and diagnosis of invasive cells and tissues, and for the monitoring of the progress of therapeutic treatments.

In a preferred embodiment of this aspect of the instant invention the nucleic acid probe comprise a specifically hybridizing fragment of the γ2 chain cDNA nucleic acid sequence. In this embodiment, the nucleic acid sequence comprises all or a specifically hybridizing fragment of an open reading frame of the nucelic acid sequence for the γ2 chain (Figure 4) encoding the amino acid sequence of the γ2 chain (Figure 4). It will be understood that the term "specfically hybridizing" when used to describe a fragment of nucleic acid encoding a human laminin γ2 chain gene is intended to mean that, nucleic acid hybridization of such a fragment is stable under high stringency conditions of hybridization and washing as the term "high stringency" would be understood by those having skill in the molecular biological arts.

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Further, the instant invention provides for the therapeutic treatment of such invasive tissues by using γ2 chain or biologically active fragments thereof to interfere with the interactions between abberant γ2 chain and surrounding tissues. The instant invention also provides for the intervemtion of γ2 chain interaction with surrounding tissues by using specific anti-γ2 chain antibodies (monoclonal or polyclonal) to inhibit the γ2 chain biological activity.

The instant disclosure also allows one to ablate the invasive cell phenotypic γ2 chain expression by using genetic manipulation to "knock-out" the functional expression of the γ2 chain gene in cancer cells, or to completely "knock-out" the functional γ2 chain gene in the genome of cancer cells. Such knock-outs can be accomplished by using genetic molecular biological techniques for inserting homologous recombination into genomic DNA, targeted transposon insertion, or random insertion/deletion mutations in the genomic DNA.

The instant disclosure also allows for the therapeutic treatment of invasive cell phenotype by the inhibition of functional γ2 chain expression in targeted cells by using anti-sense technology, such methods for anti-sense production, stabilization, delivery, and therapeutic approaches are reviewed in Uhlmann et al., 1990, *Chem. Reviews* 90:543-584).

Thus the instant invention provides for a method of detection, diagnosis, prognosis, monitoring, and therapeutic treatment of invasive cell phenotypes.

The examples below are meant by way of illustration, and are not meant to be limiting as to the scope of the instant disclosure.

Example 1: Mutation in the γ2 Chain Gene LAMC2 is critical in some cases of JEB

A unique scanning strategy using RT-PCR amplification of LAMC2 sequences was devised to detect truncated forms of γ2 chain gene transcripts (Pulkkinen et al., *Nature Genetics*, 1994, 6:293-298). The 3.6 kilobase coding sequence of the LAMC2 mRNA, was reverse transcribed and amplified with eight pairs of primers, producing overlapping PCR amplimers designated A-H. The PCR products were then examined by agarose gel electrophoresis, followed by MDE heteroduplex analysis. If bands with altered mobility were detected, the PCR products were sequenced, and compared with normal sequences from unaffected family members or unrelated individuals. Intron/exon borders were identified by PCR analysis of genomic DNA, deduced by comparison with cDNA sequences.

A point mutation produces exon skipping

When a panel of five unrelated JEB patients were analysed, the primers used to amplify segment C (nt 1046-1537) produced markedly shortened band of 273 base pairs, as compared with the normal 491 base pairs. No evidence of the normal sized band was noted, suggesting that the patient was homozygous for this allele. Direct sequencing revealed that the shortened

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product resulted from the deletion of 219 base pairs corresponding to nucleotides 1184-1402 in the cDNA, thus exon 9 was deleted. The remaining nuclotide sequences within this and other PCR products did not reveal any additional mutations upon MDE analysis.

Subsequent examination of the genomic DNA revealed that the sequences for exons 8, 9 and 10 were present, however a homozygous G for A substitution at the 3' acceptor splice site at the junction of intron 8 and exon 9, abolished the obligatory splice site sequence (AG).

Examination of another patient revealed that PCR product F (nt 2248-2777) corresponding to domains I and II of the γ2 chain, was a band with altered mobility. Sequencing the abnormal product revealed a 20 bp deletion, followed by a single base pair (G) insertion in the coding region corresponding to exon 16. This mutation causes a frameshift which results in a premature stop codon 51 base pairs downstream from the deletion-insertion, predicting a truncated kalinin/laminin 5 γ2 chain terminating at residue 830.

RT-PCR and MDE analyses

RNA isolated from fibroblast cell cultures of JEB patients was used as template for RT-PCR of the LAMC2 mRNA. (Epidermal keratinocytes can also be used). cDNA was prepared from 50 μg of total RNA in a volume of 100 μL according to manufacturer's reccomendations (BRL), and oligonucleotide primers were synthesized on the basis of the cDNA sequence (Figure 4; Kallunki et al., 1992, *supra.*), to generate ~500 base pair products, which spanned the entire coding region.

For PCR amplification, 1 μL of cDNA was used as template and amplification conditions were 94 C for 5 min followed by 95 C for 45 sec, 60 C for 45 sec and 72 C for 45 sec for 35 cycles in an OmniGene thermal cycler (Marsh Scientific). Amplification was performed in a total volume of 25 μL containing 1.5 mM $MgCl_2$, and 2 U Taq polymerase (Boehringer Mannheim). Aliquots of 5 μL were analysed on 2 % agarose gels and MDE heteroduplex analysis was performed according to the manufacturer's reccomendation (AT Biochemicals). Heteroduplexes were visualized by staining with ethidium bromide. If a band of altered mobility was detected in heteroduplex analysis, the PCR product was subcloned into the TA vector (Invitrogen), and sequenced by standard techniques.

DNA isolated either from fibroblast cultures or from specimens obtained from buccal smears, was used as template for amplification of genomic sequences. For amplification of introns 8 and 16, ~500 ng of genomic DNA was used as template and the following oligomer primers were utilized.

5' GGCTCACCAAGACTTACACA 3';

5' GAATCACTGAGCAGCTGAAC 3';

5' CAGTACCAGAACCGAGTTCG 3';

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5' CTGGTTACCAGGCTTGAGAG 3';
5' TTACTGCGGAATCTCACAGC 3';
5' TACACTGTTCAACCCAGGGT 3';
5' AAACAAGCCCTCTCACTGGT 3';
5' GCGGAGACTGTGCTGATAAG 3';
5' CATACCTCTCTACATGGCAT 3';
5' AGTCTCGCTGAATCTCTCTT 3';
5' TTACAACTAGCATGGTGCCC 3'.

Amplification conditions were 94 C for 7 min followed by 95 C for 1.5 min, 56 C (intron 8) or 58 C (intron 16) for 1 min and 72 C for 1.5 min for 35 cycles in an OmniGene thermal cycler (Marsh Scientific). Amplification was performed in a total volume of 25 μL containing 1.5 mM $MgCl_2$, and 2 U Taq polymerase (Boehringer Mannheim). The PCR products were subcloned and sequenced as above.

Verification of Mutations

The putative mutations detected in the PCR products were verified at the genomic level in both cases. For this purpose, a search for a potential change in restriction endonuclease sites as a result of the mutation was performed.

Amplification conditions were 94 C for 7 min followed by 94 C for 1 min, 58 C for 45 sec and 72 C for 45 sec for 35 cycles in an OmniGene thermal cycler (Marsh Scientific). PCR products were analysed on 2.5% agarose gels.

The methods described allow for the screening of patients for mutations in the γ2 chain which will correlate with JEB. As demonstrated, the results have identified a homozygous point mutation resulting in exon skipping, and a heterozygous deletion-insertion mutation. This demonstrating the effective screening for, and identification of, γ2 chain mutations which correlate with JEB. The methods are thus useful for diagnosis, prenatal screening, early screening and detection, as well as detailed examination of JEB. Further, the results show that the functional role of γ2 chain expression in epithelial cells is important in determining proper intercellular connectivity, relating to the integrity of tissues and cell interactions.

Example 2: Mutation in the γ2 Chain Gene LAMC2 is Critical in H-JEB

The correlation both *in vivo* and *in vitro* of the dermo-epidermal separation in H-JEB, with deficient immunoreactivity of anti-nicein/kalinin/laminin 5 antibodies, and the separation induced by anti-nicein/kalinin/laminin 5 antibodies have made the genes encoding this protein strong candidates for the site of H-JEB mutations. In this example, it is demonstrated that the molecular defect which causes H-JEB is linked to the gene encoding nicein/kalinin/laminin 5

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γ2 chain. In particular, the occurence of a homozygous premature termination codon mutation is the specific cause in an examined case of H-JEB (Aberdam et al., *Nature Genetics*, 1994, 6:299-304).

Expression of mRNA encoding the three nicein subunits by northern analysis of RNA isolated from primary keratinocyte culture of a H-JEB patient was determined as the initial screen. Hybridization with probes for the α3 and ß3 subunits was normal, but no hybridization with a cDNA encoding the γ2 subunit was detected. Examination of the genomic DNA for gross abnormalities, such as large deletions, insertions or rearrangements, in LAMC2 (the γ2 subunit gene) by Southern blot analysis turned up no abnormalities when the genomic DNA was digested with BamHI, BglI, HindIII, PstI or PvuII and probed with full length LAMC2 cDNA.

Possible mutations in the γ2 subunit were sought by using cDNA reverse transcribed from total RNA purified from cultured keratinocytes of the H-JEB patient, and subjected to PCR amplification. The size of the amplified products was checked by electrophoresis on 2% agarose gels and compared with that obtained from healthy controls.

No major differences were detected in the agarose gels, and the PCR products were examined by heteroduplex analysis (MDE). Heteroduplex analysis of the most 5' PCR product (nt 35-726) revealed the presence of a homoduplex in the proband (pateint) and the controls. However, when the amplified PCR products from the patient and control were mixed together, an additional band with altered mobility, representing heteroduplexes, was detected, suggesting a homozygous mutation in the patient's LAMC2 cDNA (Figure 5a). This amplified fragment corresponded to domain V of the γ2 protein (Vailly et al., *Eur. J. Biochem.*, 1994, 219:209-218). Sequencing detected a C to T transition at position +283, leading to a nonsense mutation in which a termination codon TGA replaces an arginine (CGA), perhaps arising as a result of the hypermutability of 5-methyl-cytosine to thymine at CpG nucleotides. This mutation, R95X, leads to truncation of the γ2 subunit polypeptide at amino acid 95 and loss of a TaqI restriction site (TCGA). Digestion of cDNA with TaqI confirmed the presence of a homozygous mutation in the DNA of the H-JEB patient. No other mutations were detected.

To confirm the cosegregation of the mutation with the loss of the TaqI restriction site, eight genotyped individuals of the family of the patient were screened. In each case, a 120 base pair fragment was amplified by PCR using genomic DNA templates and primers flanking the restriction site. Upon digestion of the wild type amplification product, two clevage fragments of 80 and 40 base pairs are generated. Consistent with the presence of a heterozygous mutation in carriers of this genotype, DNA fragments of 120, 80 and 40 base pairs, indicative of a wild type genotype, were found in the paternal grandmother and two other relatives.

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Cell Culture

Epidermis was separated from dermis by dispase treatment at 37 C. Keratinocytes were dissociated in 0.25% trypsin at 37 C and plated onto a feeder layer of irradiated mouse 3T3 cells (ICN) (Rheinwald & Green, *Cell*, 175, 6:331-334). Keratinocytes were grown in a 1:1 mixture of DMEM and Ham's F12 (BRL) containing 10% Fetal Calf Serum (FCS), 1 mM sodium pyruvate, 2 mM L-glutamine, 10 µg/mL of penicillin and strptomycin, 10 ng/mL transferrin, 180 µM adenine and 20 pM T3 (Simon & Green, *Cell*, 1985, **40**:677-683). H-JEB keratinocytes were expanded after gentle dissociation in 0.05% trypsin, 0.02% EDTA.

Northern Blot Analysis

Total RNA was prepared from H-JEB and normal cultured keratinocytes according to standard methods (Chomzynski & Sacchi, *Anal. Biochem.*, 1987, **162**:156-159). RNA was electrophoresed in 1.2% denaturing agarose gels containing 1.2 M formaldehyde and transferred onto Hybond N membrane (Amersham). Membranes were hybridized at high stringency with P-32 labeled cDNA probes corresponding to the different chains of nicein, and then exposed on Hyperfilm MP (Amersham) with intensifying screens. Radiolabeled cDNA probes NA1 (Baudoin et al., *J. Invest. Dermatol.*, 1994, *in press*), KAL-5.5C (Gerecke et al., *Eur. J. Biochem.*, 1994, *in press*), and PCR 1.3 (Vailly et al., 1994, *supra.*), were used to detect the mRNAs for nicein chains $\alpha 3$, $\beta 3$ and $\gamma 2$, respectively.

RT-PCR and heteroduplex analysis (MDE)

50 µg of total RNA isolated from cultured keratinocytes from JEB patient, and unrelated healthy controls were reverse transcribed in a volume of 100 µL as recommended by the manufacturer (BRL). 1 µL of the reaction product was used to amplify overlapping regions of the cDNA that spanned the open reading frame. Primer pair used to identify the mutation R95X: (L) 5'-GAGCGCAGAGTGAGAACCAC-3', (R) 5'-ACTGTATTCTGCAGAGCTGC-3'. PCR cycling conditions were: 94 C, 5 min, followed by 94 C, 45 sec; 60 C, 45 sec; 72 C, 45 sec; for 35 cycles, and extension at 72 C for 5 min. 5 µL aliquots were run in 2% agarose gels. Heteroduplex analysis was performed as recommended by the manufacturer (MDE, AT Biochemicals). Heteroduplexes were visualized under UV light in the presence of ethidium bromide and photographed. Amplified cDNA fragments with altered mobility were subcloned into the TA vector according to the manufacturer's recommendations (Invitrogen). Sequence analysis were then performed using standard techniques.

Verification of the mutation

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PCR reactions on genomic DNA (50 µg) were carried out using the upstream primer 5'-TTCCTTTCCCCTACCTTGTG-3' and the downstream primer 5'-TGTGGAAGCCTGGCAGACAT-3', which are located in the intron 2 and exon 3 of LAMC2 respectively. PCR conditions were: 95 C, 5 min, followed by 94 C, 45 sec; 56 C, 45 sec; 72 C, 45 sec; for 35 cycles, and extension at 72 C for 5 min. PCR products were used for restriction analysis. 20 µL of PCR produc vith TaqI for 2 hours (Boehringer Mannheim) % agarose) stained and visualized under UV light.

Thus the methods allo· ı the γ2 chain which correlate with H-JEB. A ıonsense mutation resulting in a truncated γ2 chai onfirmed by specific amplification and resu ı relatives. Thus demonstrating the effective sci tations which correlate with H-JEB. The mei :reening, early screening and detection, as we ıore, the results demonstrate the significance o ıcts.

Example 3: γ2 Chain as Diagnostic for Invasive Tissues

In this example, *in situ* hybridization is used to demonstrate the expression of the kalinin/laminin 5 γ2 chain in a variety of human cancer tissues and in skin wound healing in mice (Pyke et al., *Amer. J. Pathol.*, Oct. 1994, **145**(4):1-10 *in press*).

Thirty-six routinely processed, formalin-fixed and paraffin wax-embedded specimens from cancer surgery performed from 1991 to 1993 were drawn from pathology department files at Herlev Hospital (Copenhagen, Denmark). The specimens were evaluated according to standard criteria and included 16 cases of moderately or well-differentiated colon adenocarcinomas, 7 cases of ductal mammary carcinomas, 4 squamous cell carcinomas (2 skin, 1 cervix, 1 vulva), 3 malignant melanomas, and 6 sarcomas (3 leiomyosarcomas, 2 malingnant fibrous histiocytomas, 1 neurofibrosarcoma).

All samples were selected upon histological examination of a hematoxylin and eosin-stained section to ensure that they showed a well preserved morphology throughout and contained representative areas of both cancerous tissue and surrounding apparently normal, unaffected tissue. The broad zone separating these two tissue compartments is referred to as the invasive front in the following. No estimation of the effect of variations in fixation conditions was attempted, but in a previous study of plasminogen activating system components using specimens of colon adenocarcinomas collected using the same procedures, very little variation in relative mRNA levels was found (Pyke, C. PhD. Thesis, 1993, University of Copenhagen,

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Denmark). In addition, tissue from incisionally wounded mouse skin prepared as described by Romer et al. (*J. Invest. Dermatol.*, 1994, **102**:519-522), was fixed and paraffin-embedded the same way as the human cancer specimens.

For preparation of total RNA from six samples of colon adenocarcinomas, tissues were snap-frozen in liquid nitrogen immediately following resection and RNA was prepared as described by Lund et al., (*Biochem. J.*, 1994, *in press*).

Probes:

Fragments of the cDNA for the γ2 chain of human kalinin/laminin 5 was inserted into RNA transcription vectors by restriction enzyme cutting of clone L15 covering base pairs 2995 to 3840 (Figure 4; Kallunki et al., 1992, *supra.*). In brief, plasmids phb2t-01 and phb2t-02 were prepared by insertion of the complete L15 γ2 chain cDNA in sense and anti-sense orientation into the polylinker of plasmid vectors SP64 and SP65 (both Promega, Madison, WI), respectively. In addition, two non-overlapping fragments of clone L15 were bluntend cloned into the EcoRV-site of pKS(Bluescript)II(+) (Stratagene, La Jolla, CA) transcription vector and the resulting plasmids were verified by dideoxy sequencing according to Sanger et al (*PNAS(USA)*, 1977, **74**:5463-5471). Plasmid phb2t-03 cover bases 3003-3239 and phb2t-05 cover bases 3239 to 3839, numbers referring to cDNA sequence Z15008 in the EMBL/GenBank/DDBJ database as reported by Kallunki et al., (1992, *supra.*; Figure 4).

Similarly, cDNA fragments of other human laminin chains were prepared in RNA transcription vectors, yielding the following plasmid constructs (numbers in brackets refer to base pair numbers in the EMBL/GenBank/DDBJ sequence database by the listed accession numbers); chain α1: plasmid phae-01 (3244-3584 (accession No. X58531, Nissinen et al., *Biochem. J.*, 1991, **276**:369-379) in pKS(Bluescript)II(+)); chain β1: plasmid phb1e-01 (3460-4366 (accession No. J02778, Pikkarainen et al., *J. Biol. Chem.*, 1987, **262**:10454-10462) in pKS(Bluescript)II(+)); chain γ1: plasmids A1PSP64 and A1PSP65 (919-1535 (accession No. M55210, Pikkarainen et al., *J. Biol. Chem.*, 1988, **263**:6751-6758) in SP64 and SP65 repectively (sense and anti-sense orientation)).

All plasmids were linearized for transcription using restriction endonucleases and 5 μg of the linearized plasmids was extracted with phenol and with choloroform/isoamyl alcohol (25:1), precipitated with ethanol, and redissolved in water. Each transcription reaction contained 1 μg linearized DNA template, and transcriptions were performed essentially as recommended by the manufacturer of the polymerases. The RNA was hydrolyzed in 0.1 mol/L sodium carbonate buffer, pH 10.2, containing 10 mmol/L dithiothreitol (DTT) to an average size of 100 bases. RNA probes transcribed from opposite strands of the same plasmid template,

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yielding sense and anti-sense transcripts, were adjusted to $1x10^6$ cpm/μL and stored at -20 C until used. Probes were applied to tissue sections.

In situ Hybridization:

In situ Hybridization was performed as described by Pyke et al., (*Am. J. Pathol.*, 1991, 38:1059-1067) with S^{35} labeled RNA probes prepared as described above. In brief, paraffin sections were cut, placed on gelatinized slides, heated to 60 C for 30 minutes, deparaffinized in xylene, and rehydrated through graded alcohols to PBS (0.01 mol/L sodium phosphate buffer, pH 7.4, containing 0.14 mol/L NaCl). The slides were then washed twice in PBS, incubated with 5 μg/mL proteinase K in 50 mmol/L Tris/HCl, pH 8.0, with 5 mmol/L EDTA for 7.5 minutes, washed in PBS (2 minutes), dehydrated in graded ethanols, and air-dried before the RNA probe (~80 pg/μL) was applied. The hybridization solution consisted of deionized formamide (50%), dextran sulfate (10%), tRNA (1 μg/μL), Ficoll 400 (0.02% (w/v)), polyvinylpyrrolidone (0.02% (w/v)), BSA fraction V (0.02% (w/v)), 10 mmol/L DTT, 0.3 M NaCl, 0.5 mmol/L EDTA, 10 mmol/L Tris-HCl, and 10 mmol/L $NaPO_4$ (pH 6.8). Sections were covered by alcohol-washed, autoclaved coverslips and hybridized at 47 C overnight (16 to 18 hours) in a chamber humidified with 10 ml of a mixture similar to the hybridization solution, except for the omission of probe, dextran sulfate, DTT, and tRNA (washing mixture). After hybridization, slides were washed in washing mixture for 2 x 1 hour at 50 C, followed by 0.5 mol/L NaCl, 1 mmol/L EDTA, 10 mmol/L Tris-HCl (pH 7.2) (NTE) with 10 mmol/L DTT at 37 C for 15 minutes. After treatment with RNAse A (20 μg/mL) in NTE at 37 C for 30 minutes, the sections were washed in NTE at 37 C (2 x 30 minutes), and in 2 L of 15 mmol/L sodium chloride, 1.5 mmol/L sodium citrate, pH 7.0, with 1 mmol/L DTT for 30 minutes at room temperature with stirring. Sections were then dehydrated and air-dried. Finally, autoradiographic emulsion was applied according to the manufacturer's reccomendations, and sections were stored in black airtight boxes at 4 C until they were developed after 1 to 2 weeks of exposure.

Results : Laminin α1, β1, γ1, and γ2 chains

All rounds of *in situr* hybridization include both sense and anti-sense RNA probes for each of the genes studied. As negative controls, sense RNA probes are applied to adjacent sections and these probes consistently are negative. As a positive control of the γ2 chain hybridizations, two anti-sense probes derived from non-overlapping γ2 chain cDNA clones are used on a number of sections. To summarizes the γ2 chain expression found; all carcinomas were positive except for one case of mammary duct carcinoma, and all three cases of leiomyosarcomas, both cases of malignant fibrous histiocytoma, and the only case of

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neurofibrosarcoma. The positive controls always give similar staining on adjacent sections (see Figure 2, E and G). Fifteen of the malignant cases and all mouse tissue blocks were hybridized on two or more separate occasions giving the same hybridization pattern. All cell types other than those described below were negative in all cases.

Colon Adenocarcinoma

Sixteen specimens of colon adenocarcinoma were investigated by *in situ* hybridization for expression of the γ2 chain (Figure 1). In all of these cases, mRNA for γ2 chain was present exclusively in cancer cells and in most of the cases, staining was confined to a distinct subpopulation of cancer cells at the invasive front (Figure 1, A-D). A characteristic feature of γ2 chain containing cancer cells at the invasive front was that they appeared to represent cells in the process of branching or dissociating from larger well differentiated epithelial glands, a phenomenon referred to in the literature as tumor budding or tumor-cell dissociation.

In normal-looking colon mucosa distal from the invasive carcinoma, moderate signals for γ2 chain mRNA were observed in two specimens in the epithelial cells of a few mucosal glands that showed clear morphological signs of glandular disintegration and phagocytic cell infiltration. Apart from this, a weak signal was seen in luminal epithelial cells in normal looking colon mucosa in most specimens.

Weak signals for laminin chains α1, ß1, and γ1 mRNAs were detected in cancerous areas of the 6 colon cancers studied for the expression of these genes. The expression of each of the three genes showed a similar distribution. Expression in stromal cells with a fibroblast-like morphology as well as in endothelial cells of smaller vessels was consistently found . In marked contrast to the γ2 chain expression in the same samples, expression of α1, ß1, or γ1 was never found in cancer cells and no correlation between expression of α1, ß1, and γ1 chains with sites of invasion was found. Adjacent normal-looking parts of the samples were negative or only weakly positive for these laminin chains.

Figure 1 shows In situ hybridization of a specimen of colon adenocarcinoma for γ2 chain mRNA using a S-35 labeled anti-sense RNA probe derived from plasmid pbb2r-02. Figure 1A is a cluster of heavily labeled cancer cells at the invasive front (open arrow) in close proximity to a well-differentiated glandular structure (straight arrow). Figure 1B shows a high-magnification view of the area at the open arrow in 1A. Note that the isolated cancer cells show prominent labeling, whereas many coherent cancer cells of an adjacent glandular structure are negative (straight arrow). Figure 1C shows the same pattern at an invasive focus in another part of the same specimen. Figure 1D shows strong γ2 chain expression in cancer cells engaged in a bifurcation process (curved arrows). The malignant glandular epithelium from which the γ2

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chain-positive cancer cells are branching is negative (straight arrow). Magnification: 1A x 100; 1B-1D x 640.

Ductal Mammary Carcinomas

Six of the seven cases showed a prominent signal for γ2 chain in a small subpopulation of cells intimately associated with invasively growing malignant glandular structures. The most prominent signal was seen in cells located at the border between malignant and surrounding stromal tissue in glandular structures that exhibited clear histological signs of active invasion (Figure 2A). On careful examination it was concluded that the majority of the positive cells were cancer cells but it was not possible to determine if the cells of myoepithelial origin were also positive in some cases. One case was totally negative. Normal-appearing glandular tissue was negative in all cases.

Weak signals for laminin chains α1, ß1, and γ1 mRNAs were detected in fibroblast-like stromal cells throughout cancerous areas in one of the cases.

Malignant Melanoma

In all three cases strong hybridization of γ2 chain was found in a population of cancer cells in the radial growth phase (Figure 2B). Laminin chains α1, ß1, and γ1 were weakly expressed in the endothelium of small vessels and in fibroblast-like stromal cells throughout the affected areas in the two cases studied for these components. In addition, a weak signal for these chains was seen in sebaceous glands of adjacent normal skin.

Squamous Cell Carcinomas

In all four squamous cell carcinomas investigated, the same pattern of γ2 chain expression was found as in other carcinomas. The signals were found only in cancer cells, and only in areas with signs of ongoing invasion (Figure 2, C-G).

The four cases were also studied for mRNA of α1, ß1, and γ1 chains. In the two skin cancers, it was found that a very weak signal occured in malignant cells, and that the weak signal was in all cancer cells and of an equal intensity. This is in clear contrast to the pattern of expression of the γ2 chain. As seen in melanomas, epithelial cells of sebaceous glands present in adjacent unaffected skin were weakly positive for these laminin chains. In the other two cases (cervix and vulva) weak expression of α1, ß1, and γ1 chains were seen only in endothelial and fibroblast-like stromal cells throughout the cancerous areas (Figure 2F).

Figure 2 shows In situ hybridization for γ2 chain mRNA on sections of ductal mammary carcinoma (2A), malignant melanoma (2B), squamous cell carcinoma of the skin (2C-2D), and squamous cell carcinoma of the vulva (2E-2G). In 2A, cancer shows prominent

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signal for γ2 chain mRNA in cells bordering the zone between malignant glandular tissue and surrounding mesenchyme (curved arrows). Cancer cells located more centrally in individual malignant glandular structures are negative for γ2 chain mRNA (straight arrows). Note the wedge shaped form of the invading glandular tissue. (All images marked X' are darkfield images of the respective sections). Figure 2B shows γ2 chain mRNA signal in a subpopulation of cancer cells of radially growing malignant epithelium (curved arrows). Adjacent malignant epithelium showing a different growth pattern is devoid signal (straight arrow). Figure 2C shows γ2 chain mRNA containing cancer cells at the invasive front (curved arrow). Note lack of signal in non-invasive areas of the tumor and in adjacent unaffected areas (straight arrow). Figure 2D is a higher magnification of area of curved arrow of 2C highlighting the prominent signal in invading cells (curved arrow). Adjacent cancer cells with tumor islets are negative (straight arrow). Figure 2E shows a strong signal for γ2 chain mRNA is seen in invading cancer cells, using an anti-sense RNA probe derived from plasmid pb2t-03 (curved arrow). A postcapillary venule is negative (straight arrow). Figure 2F is a near adjacent section hybridized for laminin γ1 chain. Note that the endothelial cells of the venule show signal (straight arrow) whereas the malignant epithelium is negative (curved arrow). Figure 2G is another near-adjacent section which was hybridized for γ2 chain expression using an anti-sense RNA probe derived from a cDNA plasmid non-overlapping with that used for preparing the probe in 2E (phb2t-05). Note that the hybridization patter is similar to that seen in 2E, with strong signal in invading cancer cells (curved arrow) and absence of signal in a vessel (straight arrow). Magnification: 2C x 100, all others x 640.

Sarcomas

All six sarcomas tested in the study were totally negative for γ2 chain mRNA. The expression of other laminin chains was not done.

Mouse Wounded Skin

To compare the gene expression of γ2 chain in cancer tissue with a nonmalignant condition known to contain actively migrating epithelial cells showing a transient invasive phenotype, we hybridized sections of incisionally wounded mouse skin with γ2 chain sense and anti-sense RNA probes. Weak γ2 chain expression was observed in the keratinocytes at the edge of 12-hour old wounds, and at later time points (1-5 days), strong signals for γ2 chain mRNA was seen exclusively in the basal keratinocytes of the epidermal tongue moving under the wound clot (Figure 3). In adjacent normal-looking skin, keratinocytes were negative for γ2 chain mRNA.

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Figure 3 is incisionally wounded mouse skin (72 hours after wounding) showing signal for γ2 chain in keratinocytes at the leading edge of the migrating epithelium (curved arrow). Whereas buccal keratinocytes located more distant to the site of injury show little or no signal (straight arrow). Note that the signal for γ2 chain stops at the tip of invading keratinocytes (open arrow). A' is a dark field image of 2A. Magnification: x 640.

RNAse Protection Assay

Plasmid phbt-03 was linearized with EcoRI and a radiolabeled RNA-anti-sense probe was prepared by transcription using P-32 UTP and T3 polymerase (Pyke et al., *FEBS Letters*, 1993, **326**:69-75). RNAse protection assay, using 40 μg ethanol-precipitated and DNAse I-treated total RNA from six samples of colon adenocarcinomas was performed as described in Pyke et al., (1993, *supra.*). Protected mRNA regions were analyzed on a denaturing polyacrylamide gel and autoradiography.

The RNAse protection assay carried out on total RNA from the six samples confirmed the presence of genuine γ2 chain mRNA in all samples.

These results clearly demonstrate the important correlation of γ2 chain expression and invasive cell phenotype *in vivo*, as detected *in vitro*. Thus the instant methods present a novel and important method for the specific identification of invasive cell phenotypes in biopsied tissues. The knowledge of any information diagnostic for the presence or absence of invasive cells is useful for the monitoring and prognosis of continuing anti-carcinoma therapies. Further the identification of the expression or non-expression of the γ2 chain provides important information as to the phenotypic nature of the tissue examined. Thus the instant example demonstrates the use of probes of γ2 chain for detection of the presence, or absence, of invasive cells.

Those skilled in the art will know, or be able to ascertain, using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. These and all other equivalents are intended to be encompassed by the following claims.

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